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Altered microRNAs expression profiling in mice with diabetic neuropathic pain

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ABSTRACT

Neuropathic pain is one of the most common chronic complications of diabetes mellitus, one hallmark of which is tactile allodynia. However, the molecular mechanisms underlying tactile allodynia are not well understood. It has been demonstrated that microRNAs (miRNAs) are essential regulators of gene expression in the nervous system where they contribute to neuronal plasticity. Thus, in this study, we investigated the differentially expressed microRNAs in the lumbar spinal dorsal horn of streptozotocin (STZ)-induced diabetic neuropathic pain (DNP) mice and vehicle controls. Results from miRNA microarrays showed that 42 miRNAs were significantly altered in DNP spinal cord tissue ($P < 0.05$, fold change: $\geqslant 2$) compared with control sample. Among them, 21 miRNAs were significantly up-regulated while the other 21 down-regulated. Further validation by quantitative real-time polymerase chain reaction (qRT-PCR) indicated that the 2 significant differentially expressed candidate miRNAs (miR-184-5p and miR-190a-5p) in DNP tissue showed the same changes as in the microarray analysis. The bioinformatics analysis revealed that some of the differentially expressed miRNAs after DNP were potential regulators of some inflammation associated with genes that are known to be involved in the pathogenesis of DNP. These findings suggest that aberrant expression of miRNAs may contribute to the pathogenesis of DNP and are potential targets for therapeutic interventions following DNP.

1. Introduction

Diabetic neuropathy is one of the most common complications of diabetes mellitus [1–4]. Among the multiple symptoms of diabetic neuropathy, DNP is the most devastating complication of diabetes [5–10]. Similar to other types of neuropathic pain, patients with DNP experience a variety of aberrant sensations including spontaneous pain, hyperalgesia (increased pain responses to normally noxious stimuli) and tactile allodynia (abnormal hypersensitivity to normally innocuous stimuli) [11,12]. Nowadays DNP is regarded as the most difficult pain and is refractory to currently available treatments [11,13,14], so it is essential to elucidate the underlying mechanisms responsible for DNP.

microRNAs (miRNAs) are small noncoding RNAs, with a length of approximately 23 nucleotides, which control the expression of target mRNAs by binding to the "seed sequences" in 3′ untranslated regions (UTRs). Several studies suggest that miRNAs are involved in many disease states and biological processes where both increased and decreased expression of miRNAs have been described [15–17]. Emerging evidence indicates that some miRNAs are implicated in the regulation of genes responsible for neural plasticity and pain sensitization [18–20]. Moreover, it has been demonstrated that miRNAs are involved in pain processing based on some findings in a wide range of experimental models, such as carrageenan- or complete Freund's adjuvant (CFA)-induced inflammatory pain [21–25], spinal nerve ligation (SNL) [26,27], chronic constriction injury (CCI) [28–31], spinal cord injury [32–34]. Recently, altered expression of miRNAs have been reported in patients suffering from painful disorders such as complex regional pain syndrome [35], Fibromyalgia [36] and Osteoarthritis [37] in both affected tissues and the circulation. Accordingly, it is believed that dysregulated miRNAs may be involved in the post-transcriptional modulation of genes implicated in pain generation and maintenance [38]; however, there is no report whether the expression of miRNAs in the spinal cord were affected during the development of DNP to date. Therefore, in the present study, we

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investigated the expression of miRNAs in mice with DNP using microarray analysis.

2. Materials and methods

2.1. Animals

Male Balb/c mice aged 8 weeks were used. Mice were housed in separated cages and the room was kept at 24 ± 1 °C temperature and 50–60% humidity, under a 12:12 light–dark cycle and with free access to food and water ad libitum. All experimental procedures were approved by the Local Animal Care Committee and were carried out in accordance with the guidelines of the National Institutes of Health on animal care and the ethical guidelines for investigation of experimental pain in conscious animal [39].

2.2. Diabetic neuropathic pain model

Diabetes was induced by a single intraperitoneal (i.p.) injection of STZ (Sigma Aldrich, St. Louis, MO, USA)) at a dose of 200 mg/kg body weight after an overnight fast [6]. STZ was dissolved in 3 mM citrate buffer (pH 4.5) immediately before injection. Control mice were injected with citrate buffer solution only. Mice with blood glucose levels above 300 mg/dl were considered diabetic and used in this study. Mice were left for 42 days following STZ injection to allow for the development of neuropathic changes in diabetic mice.

2.3. Behavioral tests

Animals were habituated and basal pain sensitivity was tested before drug administration. Mechanical sensitivity was assessed with the up-down method described previously [40,41], using a set of von Frey hairs with logarithmically incremental stiffness from 0.04 to 2.04 g (0.04, 0.07, 0.16, 0.4, 0.60, 1.0, 1.4, 2.04 g). The 0.4 g stimulus, in the middle of the series, was applied first. The observation of a positive response (paw lifting, "shaking", or licking) within 5 s of the application of the filament was then followed by the application of a thinner filament (or a thicker one if the response was negative). Tactile allodynia was indicated by a significant reduction ($P < 0.05$; Student's t test) in the paw withdrawal threshold when compared to that obtained before any manipulations.

2.4. RNA isolation

Total RNA of rat lumbar spinal dorsal horn was isolated using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. RNA quantity was determined by UV spectrophotometry (KaiAo, Beijing, China) and RNA integrity was verified by gel electrophoresis using approximately 0.5 μg of total RNA per lane.

2.5. MiRNA microarray analysis

MiRNA expression profiling was performed using the RiboArray platform (RiboBio, Guangzhou, China). In brief, 1 μg of total RNA was labeled with a Cy3 using a ULS™ microRNA Labeling Kit (Kreatech, Amsterdam, Netherlands) and hybridized on the microarray. Based on Sanger miRBase version 19.0 database, RiboBio designed 1265 specific oligos for 1281 mouse miRNA, where 1265 are non-redundant sequence. In addition, 54 RiboArray™ internal controls were used as internal controls. We also put some probes for location identify function. Control probes were replicated between 3 to 40 times.

Cluster analysis using gplots (R software package) were performed. Graphs were generated by R. After data extraction, background for individual samples was calculated. For the background calculation, their median signal intensity that can be used for subtraction was calculated. The microarray data for individual samples were normalized by a quantile normalization using the probes with signal value greater than zero, signal value lesser than 65,535 (saturation value). A total of 3789 probes were used in the final analysis. T-test P-value of <0.05 and fold-change (>2, <0.5) were applied to determine two differentially expressed sets of genes of six experimental samples (lumbar spinal dorsal horn of four mice as a sample). We also performed Hierarchical clustering based on Euclidean distance measure of samples using the normalized significant genes. We looked into the patterns of expressed changes for groups.

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

QRT-PCR was performed following the protocol of RevertAid First Strand cDNA Synthesis Kit (#K1622, Thermofisher, USA). In brief, reverse transcriptase reactions contained 1 μg of purified total RNA, 10 μM stem-loop RT primer, 5× Reaction buffer, 2.5 mM each of dATP, dGTP, dCTP and dTTP, 200 U/μl reverse transcriptase and 40 U/μl RNase inhibitor. Using the Gene Amp PCR System 9700 (Applied Biosystems, USA), 20 μl reactions were performed with the following thermal cycling parameters: 5 min at 25 °C, 60 min at 42 °C, 5 min at 70 °C, and held at 4 °C. Each reaction mixture for real-time quantitative PCR contained 10 μl Maxima® SYBR Green/ROX qPCR Master Mix (2X) (#K0223, Thermofisher, USA), 0.3 μl of each primer (10 μM) (see Table 1), 0.8 μl cDNA, and nuclease-free water to a total volume of 20 μl. Reaction were run on ABI stepone plus (Life technology, USA) with the following thermal cycling parameters: initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 15 s. U6 was used as endogenous control. The experiment was conducted in triplicate. Critical threshold (CT) values were determined using ABI Stepone Software (Applied Biosystems). The relative quantity of each miRNA to U6 was calculated using the $2^{-\Delta\Delta CT}$ method.

2.7. Predication of the potential target miRNAs of genes

To date, there is not one algorithm that outperforms others in terms of sensitivity and specificity. The potential target miRNAs

Table 1
Oligonucleotides used in the study.

Primer set name	Reverse transcriptase reaction primer (5′–3′)	Real-time quantitative PCR primer (5′–3′)
U6	5′AACGCTTCACGAATTTGCGT3′	F: 5′CTCGCTTCGGCAGCACA3′ R: 5′AACGCTTCACGAATTTGCGT3′
mmu-miR-184-5p	5′CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCTGGCT3′	GSP: 5′ACACTCCAGCTGGGGCCTTATCACTTTTCC3′ R: 5′CTCAACTGGTGTCGTGGAGTCG3′
mmu-miR-190a-5p	5′CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACCTAAT3′	GSP: 5′ACACTCCAGCTGGGGTGATATGTTTGATAT3′ R: 5′CTCAACTGGTGTCGTGGAGTCG3′

of these genes were identified by both miRWalk and other programs (miRanda, Sanger miRDB, RNAhybrid and Targetscan) in the most used prediction website (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/predictedmirnagene.html).

2.8. Statistical analysis

All data are presented as mean ± SE. Statistical significance was determined using the Student's *t* test. A *P* value <0.05 was considered to be statistically significant.

3. Results

3.1. Changes in blood glucose level and mechanical pain sensitivity in mice injected with STZ

Mice injected with 200 mg/kg STZ exhibited significantly increased blood glucose level on day 1 and the hyperglycemia persisted at least for 42 days (pre-injection, 149.1 ± 6.043; day 1 post-STZ injection, 474.75 ± 12.937; day 42 post-STZ, 548.1 ± 7.585 mg/dl, $n = 12$, $P < 0.0001$) as compared with vehicle-treated control mice (pre-injection, 146.4 ± 4.229; day 1 vehicle-control, 151.5 ± 3.985; day 42 vehicle-control, 152.7 ± 4.989 mg/dl, $n = 12$). The mice which displayed significantly decreased paw withdrawal thresholds 42 days after STZ injection were selected to the gene expression assays (pre-injection, 2.781 ± 0.355; day 42 post-STZ, 0.057 ± 0.021 g, $n = 12$, $P < 0.01$). Control mice showed no significant changes in paw withdrawal thresholds (pre-injection, 2.447 ± 0.398; day 42 vehicle-control, 2.109 ± 0.419 g, $n = 12$) (see Table 2).

3.2. miRNA expression profiling in mice with DNP

To functionally investigate a possible link between the changes of miRNA expression patterns and the development of DNP, the relative expression of spinal cord miRNAs in DNP vs control mice was analyzed using microarrays. A total of 1265 miRNAs were detected by miRNA microarray in two groups. The microarray-based experiments identified 21 up-regulated and 21 down-regulated miRNAs at least 2.0-fold in DNP samples (shown in Table 3). Expression in DNP tissues compared to control tissues was increased on average by 2.0- to 13.5-fold, but decreased by 0.05- to 0.49-fold (shown in Table 3). The *P* values for these 42 miRNAs were less than 0.05 in DNP tissue compared with control tissue.

The 42 differentially expressed miRNAs were converted into a heat map to show distinguishable miRNA expression profiling samples (Fig. 1). Each column represents a sample and each row shows miRNA. The dendrograms of clustering analysis for miRNAs and the samples are displayed on the right and the bottom respectively. The bottom dendrogram separated the expression profiles of control group from DNP group.

3.3. Validation of microarray results using qRT-PCR

In order to validate the microarray results, qRT-PCR analysis of DNP and control spinal cord tissues was performed for miR-184-5p and miR-190a-5p. Compared with control spinal cord tissues, the expression of miR-184-5p and miR-190a-5p was down-regulated in DNP ones (Fig. 2), and the trend was consistent with the microarray results.

Table 3
2.0-fold up/down changes for miRNAs in DNP tissue.

Name	Fold change	*P* value
mmu-miR-3965	13.50 ± 0.696	0.001
mmu-miR-3063-5p	4.60 ± 0.815	0.020
mmu-miR-466n-5p	3.34 ± 0.408	0.022
mmu-miR-505-5p	2.81 ± 0.227	0.011
mmu-miR-196a-2-3p	2.78 ± 0.380	0.037
mmu-miR-5710	2.77 ± 0.287	0.036
mmu-miR-466a-5p	2.75 ± 0.465	0.049
mmu-miR-466b-5p	2.54 ± 0.376	0.031
mmu-miR-3473a	2.50 ± 0.373	0.020
mmu-miR-3060-5p	2.45 ± 0.198	0.022
mmu-miR-122-5p	2.43 ± 0.229	0.034
mmu-miR-466p-5p	2.32 ± 0.253	0.021
mmu-miR-187-3p	2.27 ± 0.090	0.005
mmu-miR-128-1-5p	2.25 ± 0.205	0.026
mmu-miR-3074-2-3p	2.17 ± 0.203	0.044
mmu-miR-210-3p	2.07 ± 0.170	0.030
mmu-miR-3475	2.05 ± 0.209	0.032
mmu-miR-194-1-3p	2.04 ± 0.235	0.041
mmu-miR-27a-5p	2.03 ± 0.175	0.039
mmu-miR-667-3p	2.02 ± 0.150	0.033
mmu-miR-98-5p	2.00 ± 0.110	0.014
mmu-miR-190a-5p	0.05 ± 0.011	0.049
mmu-miR-590-3p	0.09 ± 0.096	0.029
mmu-miR-5124a	0.31 ± 0.049	0.016
mmu-miR-302b-3p	0.32 ± 0.186	0.017
mmu-miR-467e-3p	0.33 ± 0.006	0.000
mmu-miR-467g	0.34 ± 0.011	0.001
mmu-miR-669a-3p	0.36 ± 0.016	0.006
mmu-miR-467d-3p	0.37 ± 0.014	0.003
mmu-miR-669a-3-3p	0.39 ± 0.036	0.002
mmu-miR-451a	0.40 ± 0.015	0.004
mmu-miR-669f-3p	0.40 ± 0.026	0.002
mmu-miR-669e-3p	0.41 ± 0.024	0.012
mmu-miR-466i-3p	0.42 ± 0.028	0.012
mmu-miR-568	0.42 ± 0.030	0.013
mmu-miR-669p-3p	0.45 ± 0.021	0.017
mmu-miR-184-5p	0.45 ± 0.027	0.008
mmu-miR-466g	0.45 ± 0.014	0.002
mmu-miR-466q	0.46 ± 0.024	0.009
mmu-miR-669b-3p	0.48 ± 0.081	0.030
mmu-miR-467b-3p	0.49 ± 0.025	0.002
mmu-miR-6360	0.49 ± 0.032	0.013

3.4. Effects of miRNAs altered after DNP on inflammation associated with mouse genes

Several lines of evidence have shown that many proinflammatory and anti-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-10 and IL-13 play important roles in the initiation and maintenance of DNP [6,42,43], therefore, we examined whether these altered miRNAs after DNP had the effects on these mediators using miRanda,

Table 2
Changes in blood glucose level and paw withdrawal threshold in mice injected with STZ.

	Vehicle		STZ	
	Before injection	42 days after injection	Before injection	42 days after injection
Blood glucose (mg/dl)	146.4 ± 4.229	152.7 ± 4.989	149.1 ± 6.043	548.1 ± 7.585**
PWT (g)	2.447 ± 0.398	2.109 ± 0.419	2.781 ± 0.355	0.057 ± 0.021*

PWT: paw withdrawal threshold. Values are mean ± SE.
* $P < 0.01$ vs vehicle.
** $P < 0.0001$ vs vehicle.

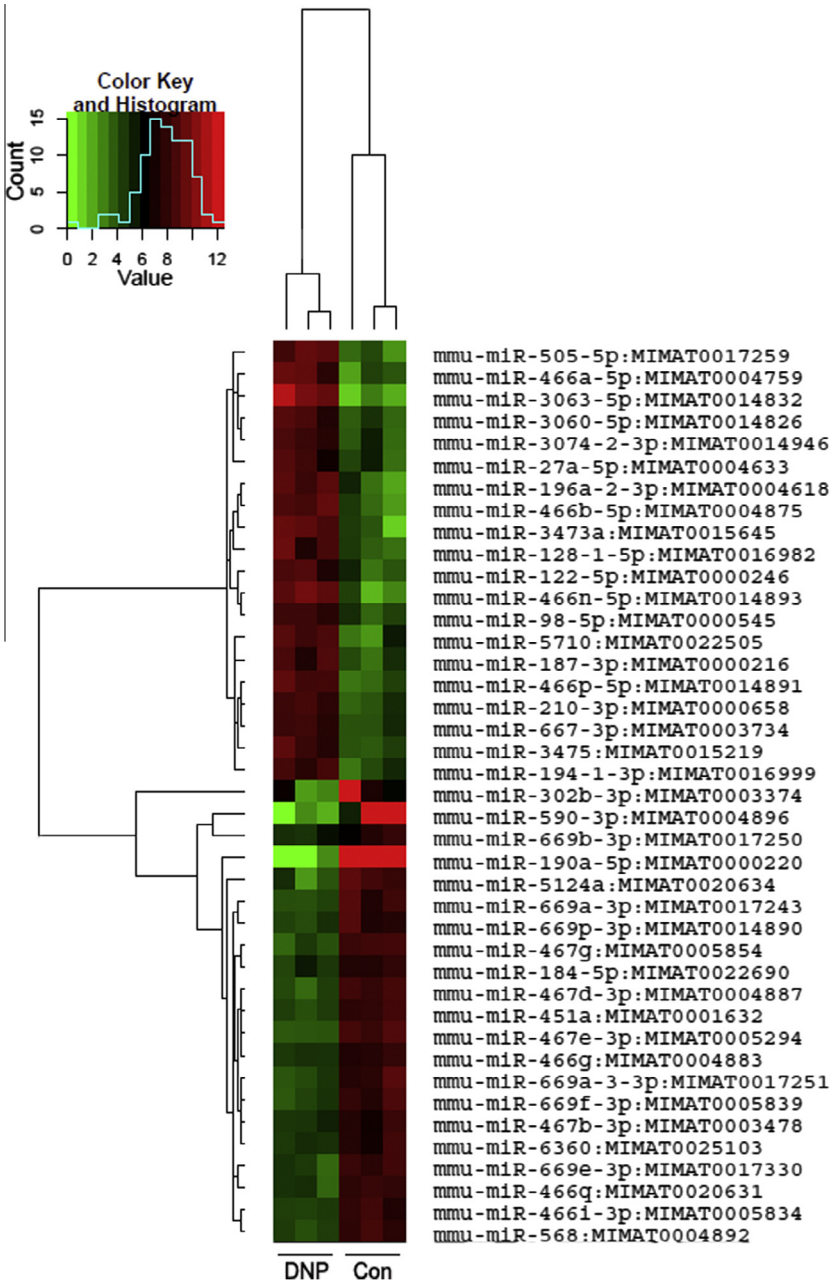

Fig. 1. Heat map shows significant expressional changes of 42 miRNAs in mice with DNP as compared with vehicle control ($n = 3$/group). The color green indicates down-regulation and red up-regulation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Sanger miRDB, miRWalk, RNAhybrid and Targetscan databases. The bioinformatics analysis indicated that miR-467b and miR-466a were potential regulators of TNF-α and IL-1β. IL-6 mRNA was potential target of miR-128, miR-194, miR-466b, miR-466c, miR-466e and miR-98. Conversely, miR-27a and miR-194 which were up-regulated after DNP were potential regulators of some antiinflammatory mRNAs such as interleukin-10 (IL-10) and interleukin-13 (IL-13), respectively. MiR-466i was a common regulator of some proinflammatory and anti-inflammatory mediators, such as TNF-α, IL-1β, IL-6 and IL-10 (shown in Table 4).

4. Discussion

In the present study, the most common experimental model of diabetes was used [6]. The blood glucose concentrations of mice significantly increased on day 1 and persisted at a high level on day 42 after STZ injection. Furthermore, the paw withdrawal thresholds were significantly reduced in DNP mice. These confirm that we duplicated the mouse DNP model successfully.

Despite increasing knowledge and ongoing research, the precise pathophysiological mechanisms of DNP remain largely unknown. Increasing evidence shows that miRNAs are dysregulated in a number of neurodegenerative diseases and play key roles in many disease-related processes [44]. There has been rarely reported on miRNA profile of DNP. Our present study firstly demonstrated obvious changes in the miRNA expression after DNP in mice. 42 differential expression miRNAs between DNP and control spinal cord tissue were found from microarray analysis. 21 miRNAs were significantly up-regulated and 21 down-regulated in DNP samples (Table 3 and Fig. 1). Two of these miRNAs were selected for further validation using qRT-PCR and the results of qRT-PCR showed that

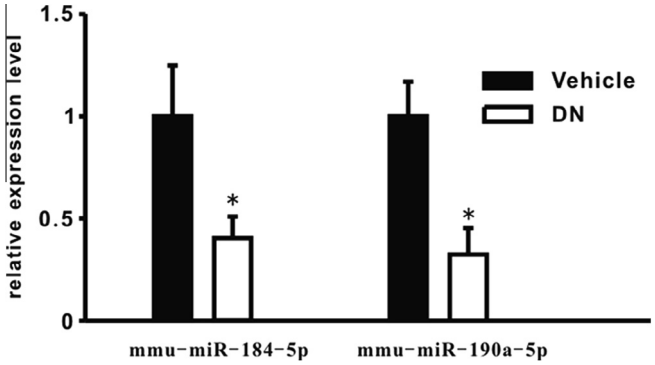

Fig. 2. Validation of differentially expressed miRNAs by qRT-PCR. qRT-PCR analysis confirmed microarray data: mmu-miR-184-5p and mmu-miR-190a-5p were down-regulated in DNP group compared to vehicle group after normalization against U6. Bars represent mean ± SE of 3 independent experiments; $^{*}P < 0.05$ vs vehicle control.

Table 4
Altered miRNAs after DNP are potential regulators of several inflammatory or anti-inflammatory genes.

Gene name	Potential target miRNA
TNF-α	mmu-miR-466i; mmu-miR-467b
IL-1β	mmu-miR-466i; mmu-miR-466a
Il-6	mmu-miR-128; mmu-miR-194; mmu-miR-466b; mmu-miR-466c; mmu-miR-466e; mmu-miR-466i; mmu-miR-98
Il-10	mmu-miR-466i; mmu-miR-27a
Il-13	mmu-miR-194

the expression level of miR-184-5p and miR-190a-5p were consist with the microarray results, therefore our microarray data were reliable. However, time-dependent changes and pathophysiological relevance of deregulation of these miRNAs after DNP need to be further validated.

A significant number of dysregulations in miRNAs have been reported in animal models of inflammatory and neuropathic pain [21–34] and our results are similar to previous studies. It has been demonstrated that peripheral nerve injury induces considerable changes of numerous genes encoding channels, receptors and signal transduction related molecules in rat spinal cord, e.g., calcium and sodium channels, neurotransmitter receptors and several protein kinases, which contribute to central sensization [45], and these modifications are likely to be controlled by miRNAs [18]. Moreover, subcutaneous inoculation of the miRNA-expressing herpes simplex virus vector against sodium channel into the feet of diabetic rats reduces pain related behaviors [46]. In the mature nervous system, increasing evidence suggests that miRNAs are crucial for normal neuronal function [18,47], thus we infer that miRNA deregulation may play an important role in the initiation and development of DNP by altering neuronal activity or enhancing neuronal excitability.

It has been demonstrated that inflammation is responsible for DNP [6,48], therefore, in the present study we have analyzed the roles of miRNAs that were altered after DNP in genes associated with these pathophysiological processes using a bioinformatics approach. The bioinformatics analysis indicates several miRNAs dysregulated after DNP are potential regulators of several inflammatory (TNF-α, IL-1β and IL-6) and anti-inflammatory genes (IL-10 and IL-13) (Table 4). In a pioneering study in 2007, it has been shown that CFA-induced inflammatory pain caused significant downregulation of miR-98 in trigeminal ganglion neurons [21]. MiR-194 has been found upregulated in a study applying the model of IL-1β-induced inflammation [49]. Consistent with these studies results, abnormal expression of miR-98 and miR-194 which are potential regulators of IL-6 have been shown in our microarray results, thus deregulation of miRNAs may affect inflammatory network homeostasis contributing to the development of DNP.

In conclusion, our study first distinguished the difference of miRNA expression profile between DNP and control mice. Furthermore, the microarray results provide a solid basis for further validation, such as identification of other miRNAs that may be related to inflammation and neuropathic pain. Demonstrating 42 altered miRNAs may be important to understand the molecular mechanisms underlying tactile allodynia during DNP. To elucidate the role of miRNAs in DNP, additional studies are required to investigate the function and targets of these miRNAs.

Conflict of interest

All authors declare that there are no conflicts of interest.

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